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TECHNICAL NOTE

Optimization of a colorimetric assay for yeast lipase activity in complex systems

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The present work describes a simple and sensitive spectrophotometric method based on the release of *p*-nitrophenyl butyrate (*p*-NPB) for the estimation of lipase activity. The method was optimized and validated in biphasic complex media containing methyl ricinoleate (MR) or olive mill wastewater (OMW), although it may be used for other biphasic media. Reaction mixtures containing substrate (2.63 mM *p*-NPB in sodium acetate buffer, 0.05 M and pH 5.6, with 4% (v/v) Triton X-100) and lipase are incubated at 37 °C during 15 minutes. After this time, 2 mL of acetone are added to stop the reaction and the corresponding absorbances are measured at 405 nm in a microplate reader. A linear response was obtained for *Candida rugosa* lipase concentrations in the range of 0.0054 to 0.1 g L^{-1} and 0.093 to 0.5 g L^{-1} for MR and OMW media, respectively. The method revealed to be more sensitive for MR medium. This conclusion was corroborated by the detection and quantification limits, which were smaller for MR than for OMW medium. The method is precise for both tested media, according to the Horwitz criterion. Besides the simplicity of this method, it is sensitive and precise, and could be adopted for routine analysis, as a tool for screening and detection of lipase activity. In addition, the fact of measuring the absorbances of the samples in a microplate reader, allows to analyze a great number of samples at the same time and to achieve a considerable time saving.

Introduction

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are one of the most important classes of industrial enzymes. These enzymes catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids.[1]

Numerous microorganisms are known to be good producers of extracellular lipases.[2] Among them, is the yeast *Yarrowia lipolytica*.[3,4] In our laboratory we have been exploring the abilities of this yeast in different fields, such as the optimization of the biotechnological production of γ-decalactone (a peach-like aroma compound) through the biotransformation of ricinoleic acid[5] and the use of olive mill wastewaters (OMW) to produce lipases while degrading this waste.[6,7]

In the aroma production field, methyl ricinoleate (MR) is used as substrate and source of ricinoleic acid. It is known that methyl esters may induce lipase production[8] and since the presence of these enzymes in the medium may improve the availability of the substrate to the microorganism, it is important to monitor lipase activity in the process. Moreover, since the valorization process of OMW by *Y. lipolytica* is being conducted with the major goal of producing enzymes, mainly lipases, a fast and sensitive method is required to detect small concentrations of lipase in routine analysis for both processes.

Several methods have been developed for the determination of lipase activity[9,10] and although some procedures have been previously described for OMW media,[11,12] none of them was valid for our OMW samples, due to its dark color.

In lipolytic activity assays, the rates of lipase reaction can be measured by determining either the rate of disappearance of the substrate, the rate of clarification of emulsion or the rate of fatty acids released.[9,10] The speed and sensitivity of the assays for free fatty acids can be increased using colorimetric methods.[10] These methods are particularly attractive due to the availability of spectrophotometers in laboratories and are usually more useful for screening tests than for samples characterization, due to the rapidness of the assays. In addition, usually those methods require a small sample volume. For these reasons, spectrophotometric methods were tested, all based on the release of a yellow chromogen resulting from the hydrolysis of the *p*-nitrophenyl ester used as the substrate. These methods can be problematic to use in complex samples presenting a dark color, such as OMW samples. Nevertheless, we managed to develop a method with higher substrate/sample volume ratio, leading to a dilution of the OMW color, which allowed the determination of the lipolytic activity. Furthermore, a microplate reader was used to measure

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the absorbances of the samples, allowing the simultaneous analysis of a greater number of samples and save a considerable amount of time.

Therefore, the method herein proposed is a spectrophotometric one, where *p*-nitrophenyl butyrate (*p*-NPB) is used as the substrate, optimized to be applied in our complex biphasic media containing MR or OMW. Since reproducible results were obtained, the authors proceeded to the method validation, prior to its implementation as a routine analysis methodology for lipase activity determination. The method herein proposed was also compared with the well-known titrimetric pH-stat method.[13]

Experimental

Chemicals

Methyl ricinoleate was kindly provided by BIOTOR, India. All other chemicals were purchased from Sigma-Aldrich.

OMW was collected in three phases' olive oil mills from the northern Portugal.

Media composition and enzyme

Two different biphasic media were used: an oil-in-water emulsion with methyl ricinoleate (MR) as organic phase (6.7 g L^{-1} Yeast Nitrogen Base with amino acids, 2.5 g L^{-1} NH_4Cl, 30 g L^{-1} MR and 3 g L^{-1} Tween 80) and an olive mill wastewater (OMW) based medium (115 $\pm$ 1 g L^{-1} COD, 12.9 $\pm$ 0.7 g L^{-1} reducing sugars, 15.8 $\pm$ 0.8 g L^{-1} total lipids).

The standard lipase used was a commercial one, from *Candida rugosa* (Sigma, L1754). Enzyme concentrations in a range from 0 to 0.5 g L^{-1} were added to the media, in order to carry out the assays to test and validate the method.

Method optimization

For the optimization of the method, several parameters were tested and optimized: the substrate for the enzymatic reaction (*p*-nitrophenyl laurate or *p*-nitrophenyl butyrate), pH, surfactant concentration and reaction time. For OMW medium, due to its dark color, the substrate/sample ratio had to be adjusted. The parameters optimization was performed step by step, always considering the modifications adopted in the previous step.

Based on several assays described in the literature,[14,15,16] three distinct buffers, at different pH values, were tested in a 0.4 g L^{-1} aqueous lipase solution, in order to investigate the effect of pH in the lipolytic assay: sodium phosphate (0.05 M, pH 7.3), Tris–HCl (0.2 M, pH 7.2) and sodium acetate (0.05 M, pH 5.6).

Since Triton X-100 is considered a suitable emulsifier for lipase activity determinations,[17] it was tested in the lipolytic assay, at concentrations of 0%, 0.1%, 0.2%, 0.5%, 1%, 2% and 4% (v/v).

The time-dependent absorbance variation was monitored under the optimized assay conditions. Each reaction mixture was prepared in the same manner and incubated at 37 °C. The absorbance values were determined at different time points.

The substrate/sample ratio initially proposed was 950 μL/50 μL. However, although this ratio worked perfectly for aqueous and MR emulsion media, the same did not occur for OMW media. In this case, it was necessary to make an adjustment in the volumes used, so different ratios were tested: 950/50, 960/40 and 980/20 in order to verify which was the most adequate ratio to use in the dark OMW media samples.

Validation procedure

The presented method was validated in terms of linearity and sensitivity, detection and quantification limits and precision, using lipase standards similar to the unknown samples analyzed routinely in MR emulsions and in OMW based media.

All statistical treatments were performed using Microsoft Excel 2007 software.

Comparison with the pH stat titrimetric method

In order to compare our spectrophotometric method with other conventional methods, samples from fermentations with *Y. lipolytica* were analyzed in terms of lipolytic activity by the well-known pH-stat method at 37 °C and pH 7.2, using a tributyrin emulsion as the substrate. Butyric acid released by the enzymatic hydrolysis of tributyrin was continuously titrated with 0.05 N NaOH using an automatic titrator (KFT Titrino, Metrohm, Switzerland). Lipase activity was calculated from the slope of the titration curve from 3 to 8 min, one activity unit being defined as the amount of enzyme which releases one micromole of butyric acid per minute. Further details on this experimental procedure can be found elsewhere.[18]

Results were compared with the activities of lipase given by the spectrophotometric method with *p*-NPB.

Results and discussion

Method optimization

Two different methyl esters, frequently reported in the literature, were tested as substrates: *p*-nitrophenyl butyrate[9,15] and *p*-nitrophenyl laurate,[9,14,19] under the same operating conditions. Since it was very difficult to dissolve *p*-nitrophenyl laurate and no reproducible results were obtained with this substrate in our samples (data not shown), *p*-NPB was selected.

During the method adaptation procedure, the effect of several other parameters was tested: pH of the substrate solution, surfactant concentration and reaction time. Due to the very dark color of OMW medium, an adjustment in the substrate/sample ratio had also to be performed.

According to data in the literature, the optimal pH value for *Y. lipolytica*[20] and *C. rugosa*[21] lipases is 7. However, a problem arouse when the pH of the reaction medium was near the optimum for the lipase tested, *i.e.*, approximately 7. The proposed method uses acetone to stop the reaction and at pH values near neutrality, the addition of acetone results in the formation of precipitates, causing an intense turbidity, turning impossible the quantification of lipase activity by a spectrophotometric method.

Therefore, despite the tests with phosphate and Tris–HCl buffers with pH values near 7, the best results were achieved using sodium acetate buffer (0.05 M) at pH 5.6: 1151 $\pm$ 56 U L^{-1} in contrast to 389 $\pm$ 92 U L^{-1} and 110 $\pm$ 4 U L^{-1} obtained with sodium phosphate (0.05 M, pH 7.3) and Tris–HCl (0.2 M, pH 7.2), respectively. Sodium acetate buffer (0.05 M) with a pH of 5.6 revealed to be the most appropriate buffer to use in this

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lipolytic assay, so it was decided to proceed the optimization with that buffer.

The addition of acetone does not only help to stop the reaction but it also accentuates the colour of the reaction mixture, thus facilitating the visual detection of samples showing lipase activity. To further investigate this point, calibration curves were constructed with *p*-nitrophenol, in the presence and absence of acetone. It was found that the slope of the calibration curve obtained in the presence of acetone was higher than that obtained in its absence (data not shown), which suggests that the addition of acetone may also enhance the sensitivity of the method.

Lipase activity strongly depends on how the lipolytic substrate is presented to the enzyme. Since the substrate solution is a biphasic solution, the use of a surfactant to stabilize the emulsion may be of importance. When a surfactant is not employed, the lipolytic activity determination is time consuming and less reproducible.[17]

Lipase activity depends on both the type and the concentration of the surfactant used in the emulsion preparation. Among the non-ionic surfactants there are some, such as Tween, that are not suitable, since they have an ester bond which could be broken by the lipase. In this work, Triton X-100 (*p-tert*-octyl phenyl polyoxyethylene ether) was used since it has been considered a suitable emulsifier for lipase activity determinations. The favorable effect of this surfactant on lipase activity is not due to the reaction medium structure, but probably due to conformational changes in the lipase structure induced by Triton X-100, which would ease the substrate access to the lipase active site.[17]

For the new method, it was decided to test different Triton X-100 concentrations in order to verify which was the most adequate for a more accurate lipase activity determination.

According to the data displayed in Fig. 1, the use of 4% (v/v) Triton X-100 leads to more sensitive results in the determination of lipase activity, since the slope of the curves is higher with the increase of surfactant concentration. The maximum Triton X-100 concentration tested (4% v/v, *i.e.* 68.2 mM) is much higher than the CMC (0.2–0.9 mM, at 20–25 °C) and it already provides a sensitive response, thus no tests were performed with higher values.

To define the most adequate reaction time, several reaction mixtures were prepared and the lipolytic activity was determined at different reaction times.

According to the results obtained, the activity values decreased continuously during the determinations performed at times between 2 and 15 minutes (Fig. 2). After this reaction time, the lipase activity values stabilized, having no significant differences in time, after 15 minutes of reaction.

Moreover, the slope of the time *vs.* absorbance curve is constant from 2 to 35 minutes, indicating that the kinetics of the hydrolysis reaction is linear up to this time point. On the other hand, the intercept of the time *vs.* absorbance curve is higher than 0, implying that high activity values would be obtained as the time interval approaches 0. Consequently, the decline of the enzymatic activity with reaction times up to 15 minutes may reflect some imprecisions of the activity measurements when the absorbance of the samples is still similar to that of the blank rather than an actual kinetic behaviour. This problem can be overcome by considering reaction times of, at least, 15 minutes.

Considering that the proposed substrate/sample ratio of 950 μL substrate/50 μL sample was not adequate for the OMW samples, due to its dark color, distinct substrate/sample ratios were tested.

According to the results obtained (Fig. 3), the method was linear for a larger range of lipase concentrations when the ratio 980 μL substrate/20 μL sample was used.

After the optimization of all parameters, the final lipase activity method consists in a reaction mixture composed of 950 μL of substrate (2.63 mM *p*-nitrophenyl butyrate in sodium acetate buffer, 0.05 M, pH 5.6, with 4% (v/v) Triton X-100) and 50 μL of broth sample. For OMW medium, the ratio of substrate and sample should be 980 μL and 20 μL, respectively. For the blanks, medium without lipase is used as substitute of the samples.

The reaction mixture is then incubated for 15 minutes at 37 °C and it is stopped by adding 2 mL of acetone. The absorbances are

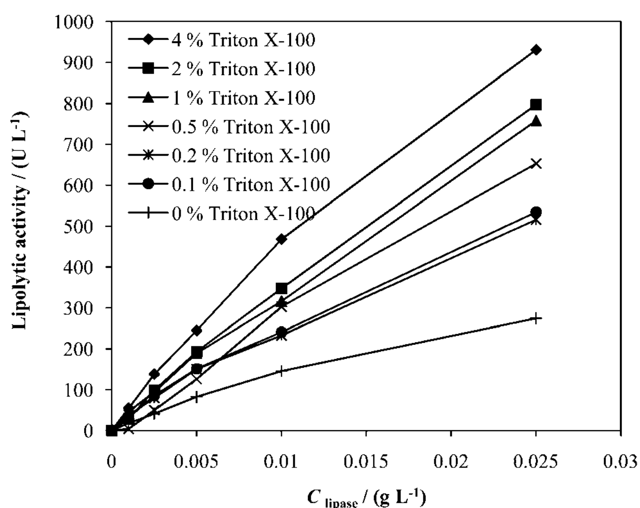

Fig. 1 The influence of surfactant concentration in the lipase method's sensitivity.

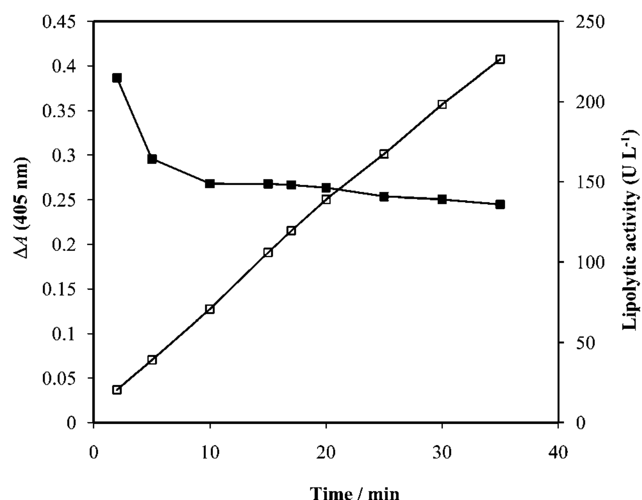

Fig. 2 The reaction time for the lipase assay: (■) lipolytic activity; (□) difference between absorbances ($A_{final} - A_{blank}$).

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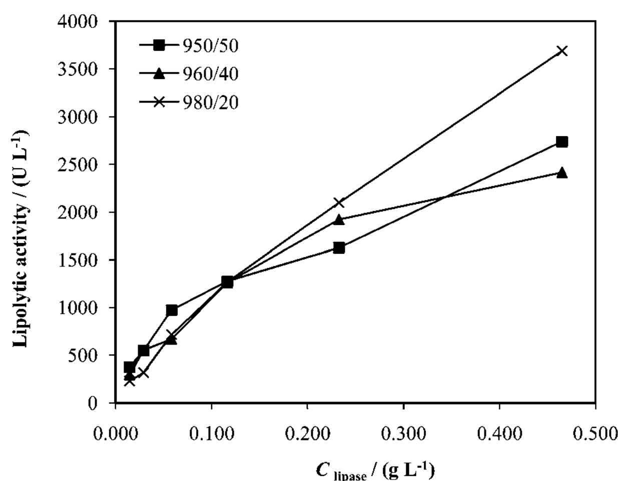

Fig. 3 The influence of different substrate/sample ratios for lipase activity determination in OMW media samples.

measured at 405 nm (OD_{405}), in a microplate reader (TECAN Sunrise, Switzerland).

One unit of activity is defined as the amount of enzyme that produces 1 µmol of *p*-nitrophenol per minute under assay conditions, and it is calculated according to eqn (1):

$$\text{Lipase Activity (U L}^{-1}) = (\Delta A/\Delta t)(1/\varepsilon)(V_t/V_s) \times 1000 \quad (1)$$

where ΔA is the difference between absorbances ($A_{\text{final}} - A_{\text{blank}}$); Δt is the time variation (min); and ε is the molar extinction coefficient of *p*-nitrophenol at 405 nm, under the same conditions of the assay (1.711 mM^{-1}); V_t is the total reaction volume (mL); and V_s is the sample volume (mL).

The molar extinction coefficient, ε, corresponds to the slope of the equation obtained by relating the absorbances measured at 405 nm with different concentrations of *p*-nitrophenol solutions prepared in sodium acetate buffer, 0.05 M, pH 5.6, with 4% (v/v) Triton X-100.

Method validation

According to ISO 8402:1994,[22] validation is the "confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled".

By carrying out a partial validation of the method, it was intended to define the range of enzyme concentrations where the method is linear, as well as the detection and quantification limits for each medium, and to be sure that the method is precise.

Linearity and sensitivity. Linearity is the ability of an analytical method to produce results directly proportional to the concentration of the analyte present in samples, in a given range of concentrations. It was defined through the relation between lipase concentration and absorbance measured at 405 nm.

Three independent assays at different enzyme concentrations (comprised in a range from 0 g L^{-1} to 0.5 g L^{-1}) were carried out and the correlation coefficient (r) was calculated by the means of least squares analysis. This parameter indicates the strength and direction of a linear relationship between two variables. The coefficient of determination (r^2) was also determined in order to verify how well the regression line represented the data.

As the deviations from linearity are often difficult to detect visually, its suitability was checked by calculating the residues between the measured and the calculated values obtained from the regression equation, performing the *t*-test. According to this statistical methodology, the calibration curves obtained from the relation between lipase concentration and the corresponding absorbance values determined at 405 nm were linear (with 95% confidence level), for the concentration ranges of 0 to 0.1 g L^{-1} and 0 to 0.5 g L^{-1} for MR and OMW based media, respectively. However, considering the quantification limits (described in the sequence), the linearity ranges were considered to be from 0.0054 to 0.1 g L^{-1} and from 0.093 to 0.5 g L^{-1} for MR and OMW based media, respectively (Table 1).

The great values (higher than 0.9992) obtained for the calculated correlation (r) and determination (r^2) coefficients for both media suggest a strong linear relationship between the two variables and also an excellent representation of the data by the regression equations of the curves obtained.

The sensitivity of an analytical method is the capability of the method to discriminate small differences in the analyte concentration. In practical terms, it can be represented by the slope of the calibration curve obtained by plotting the response against the analyte concentration.

According to the slopes from the regression equations of curves, the method revealed to be more sensitive for MR emulsions (3.76 ± 0.08) than for OMW based media (1.34 ± 0.14).

Detection and quantification limits. The limits of detection (smallest concentration of analyte in the sample that can be reliably distinguished from zero) and quantification (concentration below which the analytical method cannot operate with an acceptable precision) were calculated from the calibration lines that defined linearity, using eqn (2) and (3), according to the Long and Winefordner criterion.[23]

$$\text{DL} = (3 \times S_b)/m \quad (2)$$

$$\text{QL} = (10 \times S_b)/m \quad (3)$$

where S_b is the standard error of the independent term of the regression and m is the analytical sensitivity.

The limits of detection and quantification of the optimized method in both biphasic media (MR and OMW) are listed in

Table 1 Linearity parameters of a calibration curve obtained for the lipolytic activity determination in MR and OMW media

Parameter/media		MR	OMW
Linearity range/g L^{-1}		0.0054–0.1	0.093–0.5
Regression equation[a]	m	3.76 ± 0.08	1.34 ± 0.14
	b	−0.0019 ± 0.0045	0.025 ± 0.028
Correlation coefficient (r)		0.999	0.996
Coefficient of determination (r^2)		0.999	0.992

[a] Equation: $A_{405\ \text{nm}} = (m \pm S_m) \times C + (b \pm S_b)$, where m is the slope, b is the intercept, S_m and S_b are the standard errors associated to the slope and intercept, respectively; and C is the enzyme concentration (g L^{-1}).

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Table 2, along with the parameters used in their calculation, according to the Long and Winefordner criterion.[23]

The detection and quantification limits for lipase quantification in MR emulsions are smaller than the ones for OMW media, indicating that the method is more sensible in MR media. This was expected considering that MR emulsions do not possess the typical dark color of OMW media. Since the method used is a spectrophotometric one, which is based on the release of a yellow chromogen resulting from the hydrolysis of the *p*-nitrophenyl ester used as substrate, the darker the samples are, the more difficult it will be to detect that change in color. Therefore, the method will only detect color changes that are more pronounced, which will occur for higher lipase concentrations.

Precision. Precision is the closeness of agreement between independent test results obtained under stipulated conditions, for the same sample. It is usually specified in terms of standard deviation (SD) or relative standard deviation (RSD).

The precision of the method was analyzed in terms of repeatability by the Horwitz criterion,[24] according to which, to consider RSD acceptable in terms of precision, the RSD experimental values should be lower than the RSD values calculated by the Horwitz formula (eqn (4)).

$$\mathrm{RSD}\ (\%) = 2^{(1-0.5\ \log\ C)} \tag{4}$$

where C is the concentration expressed as power of ten.

Eight measurements were performed in MR and OMW media, containing lipase concentrations comprised in the linear range described for the enzyme in each medium and the experimental and theoretical RSD values were calculated. As example, the results obtained for lipase concentrations of 0.02 g L^{-1} and 0.12 g L^{-1}, in MR and OMW media, respectively, are presented in Table 3.

As the experimental RSD values are smaller than the theoretical ones for both media, the Horwitz criterion to consider the method precise was fulfilled. This criterion was accomplished in the whole linear range, for both media (data not shown).

Table 2 Limits of detection (DL) and quantification (QL) calculated for lipase activity determination in MR and OMW media, along with the parameters used in their calculation, according to the Long and Winefordner[23] criterion

Parameter/media	MR	OMW
Slope of linear regression/g U^{-1}	8778	7812
Standard error of the independent term	4.82	72.56
DL/g L^{-1}	0.0016	0.028
QL/g L^{-1}	0.0054	0.093

Table 3 Precision of the method for lipolytic activity determinations in MR and OMW media. The lipase concentrations used were 0.02 g L^{-1} and 0.12 g L^{-1}, for MR and OMW media, respectively

Parameter/media	MR (0.02 g L^{-1})	OMW (0.12 g L^{-1})
Mean ± SD/U L^{-1}	366 ± 9	1385 ± 56
Experimental RSD (%)	2.46	4.03
Theoretical RSD	10.19	7.78

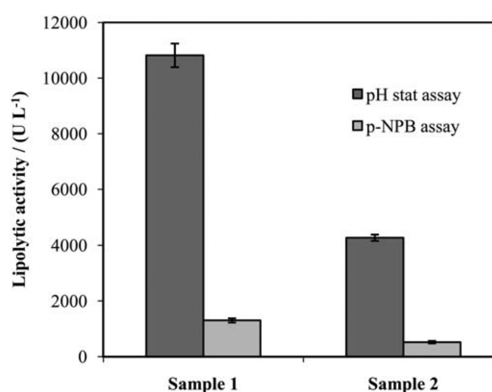

Fig. 4 Comparison between the lipolytic activities detected by titrimetric and spectrophotometric methods, in two different samples from fermentation with *Y. lipolytica*.

Comparison with a titrimetric method

Samples from fermentations with *Y. lipolytica* with different lipase activities given by our spectrophotometric method with *p*-NPB were analyzed by the well-known titrimetric pH stat method and compared with our method. The results are presented in Fig. 4.

The pH-stat method provides higher lipolytic activities than the spectrophotometric method, however, the difference between the results of each method is notably constant for both samples. This difference may be due to the higher affinity of lipase for tributyrin than for *p*-NPB. It should be mentioned that this problem is inherent to all spectrophotometric techniques for measuring lipase activity, as all of them require the use of non-natural substrates. However, the optimized method we are reporting here has a quite low detection limit (see above) and also presents some advantages over other procedures for lipase determination, such as its simplicity, low cost and the ability to process high number of samples in a rapid manner. For these reasons we consider that the proposed method is useful for screening and detection of lipase activity, for example in lipase production and purification, both at laboratory and at industrial scale. On the other hand, the pH-stat method has proven to be more suitable for kinetic studies,[18] as well as to carry out the biochemical characterization of newly isolated lipases.

Conclusions

A spectrophotometric method to determine lipase activity in biphasic complex media, based on the release of *p*-nitrophenol butyrate, has been described. The method was optimized to use in samples containing MR or OMW, and validated in these two biphasic media in terms of linearity, detection and quantification limits and precision.

Due to the good results obtained in the validation procedure and to the simplicity and rapidity of the method, it could be adopted for routine analysis to determine lipase activity in complex samples such as the ones used in this work. In addition, the fact of measuring the absorbances of the samples in

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a microplate reader allows the simultaneous analysis of a great number of samples and saves time.

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